

PATENT
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APPLICATION FOR UNITED STATES LETTERS PATENT
for
PLANTS AND SEEDS OF CORN VARIETY LH332
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BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates generally to the field of corn breeding. In particular, the invention relates to corn seed and plants of the variety designated LH332, and
5 derivatives and tissue cultures thereof.

2. DESCRIPTION OF RELATED ART

The goal of field crop breeding is to combine various desirable traits in a single variety/hybrid. Such desirable traits include greater yield, better stalks, better roots, resistance to insecticides, herbicides, pests, and disease, tolerance to heat and drought,
10 reduced time to crop maturity, better agronomic quality, higher nutritional value, and uniformity in germination times, stand establishment, growth rate, maturity, and fruit size.

Breeding techniques take advantage of a plant's method of pollination. There are two general methods of pollination: a plant self-pollinates if pollen from one flower is
15 transferred to the same or another flower of the same plant. A plant cross-pollinates if pollen comes to it from a flower on a different plant.

Corn plants (*Zea mays* L.) can be bred by both self-pollination and cross-pollination. Both types of pollination involve the corn plant's flowers. Corn has separate male and female flowers on the same plant, located on the tassel and the ear, respectively.
20 Natural pollination occurs in corn when wind blows pollen from the tassels to the silks that protrude from the tops of the ear shoot.

Plants that have been self-pollinated and selected for type over many generations become homozygous at almost all gene loci and produce a uniform population of true breeding progeny, a homozygous plant. A cross between two such homozygous plants
25 produces a uniform population of hybrid plants that are heterozygous for many gene loci. Conversely, a cross of two plants each heterozygous at a number of loci produces a population of hybrid plants that differ genetically and are not uniform. The resulting non-uniformity makes performance unpredictable.

The development of uniform corn plant hybrids requires the development of homozygous inbred plants, the crossing of these inbred plants, and the evaluation of the crosses. Pedigree breeding and recurrent selection are examples of breeding methods used to develop inbred plants from breeding populations. Those breeding methods
5 combine the genetic backgrounds from two or more inbred plants or various other broad-based sources into breeding pools from which new inbred plants are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred plants and the hybrids from these crosses are evaluated to determine which of those have commercial potential.

10 North American farmers plant tens of millions of acres of corn at the present time and there are extensive national and international commercial corn breeding programs. A continuing goal of these corn breeding programs is to develop corn hybrids that are based on stable inbred plants and have one or more desirable characteristics. To accomplish this goal, the corn breeder must select and develop superior inbred parental plants.

15 SUMMARY OF THE INVENTION

In one aspect, the present invention provides a corn plant of the variety designated LH332. Also provided are corn plants having all the physiological and morphological characteristics of the inbred corn variety LH332. The inbred corn plant of the invention
20 may further comprise, or have, a cytoplasmic or nuclear factor that is capable of conferring male sterility or otherwise preventing self-pollination, such as by self-incompatibility. Parts of the corn plant of the present invention are also provided, for example, pollen obtained from an inbred plant and an ovule of the inbred plant.

The invention also concerns seed of the inbred corn variety LH332. The inbred
25 corn seed of the invention may be provided as an essentially homogeneous population of inbred corn seed of the variety designated LH332. Essentially homogeneous populations of inbred seed are generally free from substantial numbers of other seed. Therefore, in the practice of the present invention, inbred seed generally forms at least about 97% of the total seed. The population of inbred corn seed of the invention may be particularly
30 defined as being essentially free from hybrid seed. The inbred seed population may be

separately grown to provide an essentially homogeneous population of inbred corn plants designated LH332.

In another aspect of the invention, a conversion of the corn variety LH332 is provided. The conversion may comprise a genetic locus that is a dominant or recessive allele. In certain embodiments of the invention, the genetic locus confers one or more traits such as, for example, male sterility, yield stability, waxy starch, yield enhancement, industrial usage, herbicide resistance, insect resistance, resistance to bacterial, fungal, nematode or viral disease, male fertility, and enhanced nutritional quality. The genetic locus may be a naturally occurring maize gene introduced into the genome of the variety by backcrossing, a natural or induced mutation, or a transgene introduced through genetic transformation techniques. When introduced through transformation, a genetic locus may comprise one or more transgenes integrated at a single chromosomal location.

In yet another aspect of the invention, an inbred corn plant of the variety designated LH332 is provided, wherein a cytoplasmically-inherited trait has been introduced into said inbred plant. Such cytoplasmically-inherited traits are passed to progeny through the female parent in a particular cross. An exemplary cytoplasmically-inherited trait is the male sterility trait. Cytoplasmic-male sterility (CMS) is a pollen abortion phenomenon determined by the interaction between the genes in the cytoplasm and the nucleus. Alteration in the mitochondrial genome and the lack of restorer genes in the nucleus will lead to pollen abortion. With either a normal cytoplasm or the presence of restorer gene(s) in the nucleus, the plant will produce pollen normally. A CMS plant can be pollinated by a maintainer version of the same variety, which has a normal cytoplasm but lacks the restorer gene(s) in the nucleus, and continue to be male sterile in the next generation. The male fertility of a CMS plant can be restored by a restorer version of the same variety, which must have the restorer gene(s) in the nucleus. With the restorer gene(s) in the nucleus, the offspring of the male-sterile plant can produce normal pollen grains and propagate. A cytoplasmically inherited trait may be a naturally occurring maize trait or a trait introduced through genetic transformation techniques.

In another aspect of the invention, a tissue culture of regenerable cells of a plant of variety LH332 is provided. The tissue culture will preferably be capable of regenerating plants capable of expressing all of the physiological and morphological characteristics of the variety, and of regenerating plants having substantially the same genotype as other plants of the variety. Examples of some of the physiological and morphological characteristics of the variety LH332 include characteristics related to yield, maturity, and kernel quality, each of which is specifically disclosed herein. The regenerable cells in such tissue cultures will preferably be derived from embryos, meristematic cells, immature tassels, microspores, pollen, leaves, anthers, roots, root tips, silk, flowers, kernels, ears, cobs, husks, or stalks, or from callus or protoplasts derived from those tissues. Still further, the present invention provides corn plants regenerated from the tissue cultures of the invention, the plants having all the physiological and morphological characteristics of variety LH332.

In yet another aspect of the invention, processes are provided for producing corn seeds or plants, which processes generally comprise crossing a first parent corn plant with a second parent corn plant, wherein at least one of the first or second parent corn plants is a plant of the variety designated LH332. These processes may be further exemplified as processes for preparing hybrid corn seed or plants, wherein a first inbred corn plant is crossed with a second corn plant of a different, distinct variety to provide a hybrid that has, as one of its parents, the inbred corn plant variety LH332. In these processes, crossing will result in the production of seed. The seed production occurs regardless of whether the seed is collected or not.

In one embodiment of the invention, the first step in "crossing" comprises planting, preferably in pollinating proximity, seeds of a first and second parent corn plant, and preferably, seeds of a first inbred corn plant and a second, distinct inbred corn plant. Where the plants are not in pollinating proximity, pollination can nevertheless be accomplished by transferring a pollen or tassel bag from one plant to the other as described below.

A second step comprises cultivating or growing the seeds of said first and second parent corn plants into plants that bear flowers (corn bears both male flowers (tassels) and female flowers (silks) in separate anatomical structures on the same plant). A third step comprises preventing self-pollination of the plants, *i.e.*, preventing the silks of a plant from being fertilized by any plant of the same variety, including the same plant. This is preferably done by emasculating the male flowers of the first or second parent corn plant, (*i.e.*, treating or manipulating the flowers so as to prevent pollen production, in order to produce an emasculated parent corn plant). Self-incompatibility systems may also be used in some hybrid crops for the same purpose. Self-incompatible plants still shed viable pollen and can pollinate plants of other varieties but are incapable of pollinating themselves or other plants of the same variety.

A fourth step comprises allowing cross-pollination to occur between the first and second parent corn plants. When the plants are not in pollinating proximity, this is done by placing a bag, usually paper or glassine, over the tassels of the first plant and another bag over the silks of the incipient ear on the second plant. The bags are left in place for at least 24 hours. Since pollen is viable for less than 24 hours, this assures that the silks are not pollinated from other pollen sources, that any stray pollen on the tassels of the first plant is dead, and that the only pollen transferred comes from the first plant. The pollen bag over the tassel of the first plant is then shaken vigorously to enhance release of pollen from the tassels, and the shoot bag is removed from the silks of the incipient ear on the second plant. Finally, the pollen bag is removed from the tassel of the first plant and is placed over the silks of the incipient ear of the second plant, shaken again and left in place. Yet another step comprises harvesting the seeds from at least one of the parent corn plants. The harvested seed can be grown to produce a corn plant or hybrid corn plant.

The present invention also provides corn seed and plants produced by a process that comprises crossing a first parent corn plant with a second parent corn plant, wherein at least one of the first or second parent corn plants is a plant of the variety designated LH332. In one embodiment of the invention, corn seed and plants produced by the process are first generation (F_1) hybrid corn seed and plants produced by crossing an

inbred in accordance with the invention with another, distinct inbred. The present invention further contemplates seed of an F₁ hybrid corn plant. Therefore, certain exemplary embodiments of the invention provide an F₁ hybrid corn plant and seed thereof.

5 In still yet another aspect of the invention, the genetic complement of the corn plant variety designated LH332 is provided. The phrase “genetic complement” is used to refer to the aggregate of nucleotide sequences, the expression of which sequences defines the phenotype of, in the present case, a corn plant, or a cell or tissue of that plant. A genetic complement thus represents the genetic make up of an inbred cell, tissue or plant,
10 and a hybrid genetic complement represents the genetic make up of a hybrid cell, tissue or plant. The invention thus provides corn plant cells that have a genetic complement in accordance with the inbred corn plant cells disclosed herein, and plants, seeds and diploid plants containing such cells.

 Plant genetic complements may be assessed by genetic marker profiles, and by the
15 expression of phenotypic traits that are characteristic of the expression of the genetic complement, *e.g.*, isozyme typing profiles. It is understood that variety LH332 and plants derived therefrom can be identified by many types of genetic markers such as, for example, Simple Sequence Repeat polymorphisms (SSRs) (Williams *et al.*, 1990), isozymes, Randomly Amplified Polymorphic DNAs (RAPDs), DNA Amplification
20 Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Arbitrary Primed Polymerase Chain Reaction (AP-PCR), Amplified Fragment Length Polymorphisms (AFLPs) (EP 534 858, specifically incorporated herein by reference in its entirety), and Single Nucleotide Polymorphisms (SNPs) (Wang *et al.*, 1998).

 In still yet another aspect, the present invention provides hybrid genetic
25 complements, as represented by corn plant cells, tissues, plants, and seeds, formed by the combination of a haploid genetic complement of an inbred corn plant of the invention with a haploid genetic complement of a second corn plant, preferably, another, distinct inbred corn plant. In another aspect, the present invention provides a corn plant

regenerated from a tissue culture that comprises a hybrid genetic complement of this invention.

In still yet another aspect, the present invention provides a method of producing an inbred corn plant derived from the corn variety LH332, the method comprising the steps of: (a) preparing a progeny plant derived from corn variety LH332, wherein said preparing comprises crossing a plant of the corn variety LH332 with a second corn plant; (b) crossing the progeny plant with itself or a second plant to produce a seed of a progeny plant of a subsequent generation; (c) growing a progeny plant of a subsequent generation from said seed of a progeny plant of a subsequent generation and crossing the progeny plant of a subsequent generation with itself or a second plant; and (d) repeating steps (c) and (d) for an addition 3-10 generations to produce an inbred corn plant derived from the corn variety LH332. In the method, it may be desirable to select particular plants resulting from step (c) for continued crossing according to steps (b) and (c). By selecting plants having one or more desirable traits, an inbred corn plant derived from the corn variety LH332 is obtained which possesses some of the desirable traits of corn variety LH332 as well potentially other selected traits.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS OF PLANT CHARACTERISTICS

Barren Plants: Plants that are barren, *i.e.*, lack an ear with grain, or have an ear with only a few scattered kernels.

Cg: *Colletotrichum graminicola* rating. Rating times 10 is approximately equal to percent total plant infection.

CLN: Corn Lethal Necrosis (combination of Maize Chlorotic Mottle Virus and Maize Dwarf Mosaic virus) rating: numerical ratings are based on a severity scale where 1 = most resistant to 9 = susceptible.

Cn: *Corynebacterium nebraskense* rating. Rating times 10 is approximately equal to percent total plant infection.

Cz: *Cercospora zea-maydis* rating. Rating times 10 is approximately equal to percent total plant infection.

Dgg: *Diatraea grandiosella* girdling rating (values are percent plants girdled and stalk lodged).

Dropped Ears: Ears that have fallen from the plant to the ground.

Dsp: *Diabrotica* species root ratings (1 = least affected to 9 = severe pruning).

5 **Ear-Attitude:** The attitude or position of the ear at harvest scored as upright, horizontal, or pendant.

Ear-Cob Color: The color of the cob, scored as white, pink, red, or brown.

Ear-Cob Diameter: The average diameter of the cob measured at the midpoint.

10 **Ear-Cob Strength:** A measure of mechanical strength of the cobs to breakage, scored as strong or weak.

Ear-Diameter: The average diameter of the ear at its midpoint.

Ear-Dry Husk Color: The color of the husks at harvest scored as buff, red, or purple.

15 **Ear-Fresh Husk Color:** The color of the husks 1 to 2 weeks after pollination scored as green, red, or purple.

Ear-Husk Bract: The length of an average husk leaf scored as short, medium, or long.

Ear-Husk Cover: The average distance from the tip of the ear to the tip of the husks. Minimum value no less than zero.

20 **Ear-Husk Opening:** An evaluation of husk tightness at harvest scored as tight, intermediate, or open.

Ear-Length: The average length of the ear.

Ear-Number Per Stalk: The average number of ears per plant.

Ear-Shank Internodes: The average number of internodes on the ear shank.

25 **Ear-Shank Length:** The average length of the ear shank.

Ear-Shelling Percent: The average of the shelled grain weight divided by the sum of the shelled grain weight and cob weight for a single ear.

Ear-Silk Color: The color of the silk observed 2 to 3 days after silk emergence scored as green-yellow, yellow, pink, red, or purple.

5 **Ear-Taper (Shape):** The taper or shape of the ear scored as conical, semi-conical, or cylindrical.

Ear-Weight: The average weight of an ear.

Early Stand: The percent of plants that emerge from the ground as determined in the early spring.

10 **ER:** Ear rot rating (values approximate percent ear rotted).

Final Stand Count: The number of plants just prior to harvest.

GDUs: Growing degree units which are calculated by the Barger Method, where the heat units for a 24-h period are calculated as $GDUs = [(Maximum\ daily\ temperature + Minimum\ daily\ temperature)/2] - 50$. The highest maximum daily temperature used is 86 °F and the lowest minimum temperature used is 50°F.

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GDUs to Shed: The number of growing degree units (GDUs) or heat units required for an inbred line or hybrid to have approximately 50% of the plants shedding pollen as measured from time of planting. GDUs to shed is determined by summing the individual GDU daily values from planting date to the date of 50% pollen shed.

20 **GDUs to Silk:** The number of growing degree units for an inbred line or hybrid to have approximately 50% of the plants with silk emergence as measured from time of planting. GDUs to silk is determined by summing the individual GDU daily values from planting date to the date of 50% silking.

Hc2: *Helminthosporium carbonum* race 2 rating. Rating times 10 is approximately equal to percent total plant infection.

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Hc3: *Helminthosporium carbonum* race 3 rating. Rating times 10 is approximately equal to percent total plant infection.

Hm: *Helminthosporium maydis* race 0 rating. Rating times 10 is approximately equal to percent total plant infection.

Ht1: *Helminthosporium turcicum* race 1 rating. Rating times 10 is approximately equal to percent total plant infection.

5 **Ht2:** *Helminthosporium turcicum* race 2 rating. Rating times 10 is approximately equal to percent total plant infection.

HtG: Chlorotic-lesion type resistance. += indicates the presence of Ht chlorotic-lesion type resistance; - = indicates absence of Ht chlorotic-lesion type resistance; and +/- = indicates segregation of Ht chlorotic-lesion type resistance. Rating times 10 is
10 approximately equal to percent total plant infection.

Kernel-Aleurone Color: The color of the aleurone scored as white, pink, tan, brown, bronze, red, purple, pale purple, colorless, or variegated.

Kernel-Cap Color: The color of the kernel cap observed at dry stage, scored as white, lemon-yellow, yellow, or orange.

15 **Kernel-Endosperm Color:** The color of the endosperm scored as white, pale yellow, or yellow.

Kernel-Endosperm Type: The type of endosperm scored as normal, waxy, or opaque.

Kernel-Grade: The percent of kernels that are classified as rounds.

20 **Kernel-Length:** The average distance from the cap of the kernel to the pedicel.

Kernel-Number Per Row: The average number of kernels in a single row.

Kernel-Pericarp Color: The color of the pericarp scored as colorless, red-white crown, tan, bronze, brown, light red, cherry red, or variegated.

25 **Kernel-Row Direction:** The direction of the kernel rows on the ear scored as straight, slightly curved, spiral, or indistinct (scattered).

Kernel-Row Number: The average number of rows of kernels on a single ear.

Kernel-Side Color: The color of the kernel side observed at the dry stage, scored as white, pale yellow, yellow, orange, red, or brown.

Kernel-Thickness: The distance across the narrow side of the kernel.

Kernel-Type: The type of kernel scored as dent, flint, or intermediate.

5 **Kernel-Weight:** The average weight of a predetermined number of kernels.

Kernel-Width: The distance across the flat side of the kernel.

Kz: *Kabatiella zeae* rating. Rating times 10 is approximately equal to percent total plant infection.

10 **Leaf-Angle:** Angle of the upper leaves to the stalk scored as upright (0 to 30 degrees), intermediate (30 to 60 degrees), or lax (60 to 90 degrees).

Leaf-Color: The color of the leaves 1 to 2 weeks after pollination scored as light green, medium green, dark green, or very dark green.

Leaf-Length: The average length of the primary ear leaf.

15 **Leaf-Longitudinal Creases:** A rating of the number of longitudinal creases on the leaf surface 1 to 2 weeks after pollination. Creases are scored as absent, few, or many.

Leaf-Marginal Waves: A rating of the waviness of the leaf margin 1 to 2 weeks after pollination. Rated as none, few, or many.

20 **Leaf-Number:** The average number of leaves of a mature plant. Counting begins with the cotyledonary leaf and ends with the flag leaf.

Leaf-Sheath Anthocyanin: A rating of the level of anthocyanin in the leaf sheath 1 to 2 weeks after pollination, scored as absent, basal-weak, basal-strong, weak or strong.

25 **Leaf-Sheath Pubescence:** A rating of the pubescence of the leaf sheath. Ratings are taken 1 to 2 weeks after pollination and scored as light, medium, or heavy.

Leaf-Width: The average width of the primary ear leaf measured at its widest point.

LSS: Late season standability (values times 10 approximate percent plants lodged in disease evaluation plots).

5 **Moisture:** The moisture of the grain at harvest.

On1: *Ostrinia nubilalis* 1st brood rating (1 = resistant to 9 = susceptible).

On2: *Ostrinia nubilalis* 2nd brood rating (1 = resistant to 9 = susceptible).

10 **Relative Maturity:** A maturity rating based on regression analysis. The regression analysis is developed by utilizing check hybrids and their previously established day rating versus actual harvest moistures. Harvest moisture on the hybrid in question is determined and that moisture value is inserted into the regression equation to yield a relative maturity.

15 **Root Lodging:** Root lodging is the percentage of plants that root lodge. A plant is counted as root lodged if a portion of the plant leans from the vertical axis by approximately 30 degrees or more.

Seedling Color: Color of leaves at the 6 to 8 leaf stage.

Seedling Height: Plant height at the 6 to 8 leaf stage.

20 **Seedling Vigor:** A visual rating of the amount of vegetative growth on a 1 to 9 scale, where 1 equals best. The score is taken when the average entry in a trial is at the fifth leaf stage.

Selection Index: The selection index gives a single measure of hybrid's worth based on information from multiple traits. One of the traits that is almost always included is yield. Traits may be weighted according to the level of importance assigned to them.

Sr: *Sphacelotheca reiliana* rating is actual percent infection.

25 **Stalk-Anthocyanin:** A rating of the amount of anthocyanin pigmentation in the stalk. The stalk is rated 1 to 2 weeks after pollination as absent, basal-weak, basal-strong, weak, or strong.

Stalk-Brace Root Color: The color of the brace roots observed 1 to 2 weeks after pollination as green, red, or purple.

Stalk-Diameter: The average diameter of the lowest visible internode of the stalk.

5 **Stalk-Ear Height:** The average height of the ear measured from the ground to the point of attachment of the ear shank of the top developed ear to the stalk.

Stalk-Internode Direction: The direction of the stalk internode observed after pollination as straight or zigzag.

10 **Stalk-Internode Length:** The average length of the internode above the primary ear.

Stalk Lodging: The percentage of plants that did stalk lodge. Plants are counted as stalk lodged if the plant is broken over or off below the ear.

Stalk-Nodes With Brace Roots: The average number of nodes having brace roots per plant.

15 **Stalk-Plant Height:** The average height of the plant as measured from the soil to the tip of the tassel.

Stalk-Tillers: The percent of plants that have tillers. A tiller is defined as a secondary shoot that has developed as a tassel capable of shedding pollen.

20 **Staygreen:** Staygreen is a measure of general plant health near the time of black layer formation (physiological maturity). It is usually recorded at the time the ear husks of most entries within a trial have turned a mature color. Scoring is on a 1 to 9 basis where 1 equals best.

STR: Stalk rot rating (values represent severity rating of 1 = 25% of inoculated internode rotted to 9 = entire stalk rotted and collapsed).

25 **SVC:** Southeastern Virus Complex (combination of Maize Chlorotic Dwarf Virus and Maize Dwarf Mosaic Virus) rating; numerical ratings are based on a severity

scale where 1 = most resistant to 9 = susceptible (1988 reactions are largely Maize Dwarf Mosaic Virus reactions).

Tassel-Anther Color: The color of the anthers at 50% pollen shed scored as green-yellow, yellow, pink, red, or purple.

5 **Tassel-Attitude:** The attitude of the tassel after pollination scored as open or compact.

Tassel-Branch Angle: The angle of an average tassel branch to the main stem of the tassel scored as upright (less than 30 degrees), intermediate (30 to 45 degrees), or lax (greater than 45 degrees).

10 **Tassel-Branch Number:** The average number of primary tassel branches.

Tassel-Glume Band: The closed anthocyanin band at the base of the glume scored as present or absent.

Tassel-Glume Color: The color of the glumes at 50% shed scored as green, red, or purple.

15 **Tassel-Length:** The length of the tassel measured from the base of the bottom tassel branch to the tassel tip.

Tassel-Peduncle Length: The average length of the tassel peduncle, measured from the base of the flag leaf to the base of the bottom tassel branch.

20 **Tassel-Pollen Shed:** A visual rating of pollen shed determined by tapping the tassel and observing the pollen flow of approximately five plants per entry. Rated on a 1 to 9 scale where 9 = sterile, 1 = most pollen.

Tassel-Spike Length: The length of the spike measured from the base of the top tassel branch to the tassel tip.

25 **Test Weight:** Weight of the grain in pounds for a given volume (bushel) adjusted to 15.5% moisture.

Yield: Yield of grain at harvest adjusted to 15.5% moisture.

II. OTHER DEFINITIONS

Allele: Any of one or more alternative forms of a gene locus, all of which alleles relate to one trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

5 **Backcrossing:** A process in which a breeder repeatedly crosses hybrid progeny back to one of the parents, for example, a first generation hybrid (F_1) with one of the parental genotypes of the F_1 hybrid.

Chromatography: A technique wherein a mixture of dissolved substances are bound to a solid support followed by passing a column of fluid across the solid support
10 and varying the composition of the fluid. The components of the mixture are separated by selective elution.

Converted (Conversion) Plant: Plants which are developed by a plant breeding technique called backcrossing wherein essentially all of the desired morphological and physiological characteristics of an inbred are recovered in addition to one or more new
15 trait conferred by the genetic locus transferred into the inbred *via* the backcrossing technique. By “new trait,” it is understood that the trait may or may not be naturally occurring in maize, but is added or modified with respect to the starting inbred. A genetic locus may comprise one or more genes. In the case of transgenes, one or more transgenes are commonly integrated into a host genome at a given locus. Such transgenes may
20 comprise selectable markers, enhancers, or other components of the transformation vector used.

Crossing: The pollination of a female flower of a corn plant, thereby resulting in the production of seed from the flower.

Cross-pollination: Fertilization by the union of two gametes from different
25 plants.

Diploid: A cell or organism having two sets of chromosomes.

Electrophoresis: A process by which particles suspended in a fluid or a gel matrix are moved under the action of an electrical field, and thereby separated according

to their charge and molecular weight. This method of separation is well known to those skilled in the art and is typically applied to separating various forms of enzymes and of DNA fragments produced by restriction endonucleases.

5 **Emasculate:** The removal of plant male sex organs or the inactivation of the organs with a chemical agent or a cytoplasmic or nuclear genetic factor conferring male sterility.

Enzymes: Molecules which can act as catalysts in biological reactions.

F₁ Hybrid: The first generation progeny of the cross of two plants.

10 **Genetic Complement:** An aggregate of nucleotide sequences, the expression of which sequences defines the phenotype in corn plants, or components of plants including cells or tissue.

Genotype: The genetic constitution of a cell or organism.

Haploid: A cell or organism having one set of the two sets of chromosomes in a diploid.

15 **Isozymes:** Detectable variants of an enzyme, the variants catalyzing the same reaction(s) but differing from each other, *e.g.*, in primary structure and/or electrophoretic mobility. The differences between isozymes are under single gene, codominant control. Consequently, electrophoretic separation to produce band patterns can be equated to different alleles at the DNA level. Structural differences that do not alter charge cannot
20 be detected by this method.

Isozyme typing profile: A profile of band patterns of isozymes separated by electrophoresis that can be equated to different alleles at the DNA level.

Linkage: A phenomenon wherein alleles on the same chromosome tend to segregate together more often than expected by chance if their transmission was
25 independent.

Marker: A readily detectable phenotype, preferably inherited in codominant fashion (both alleles at a locus in a diploid heterozygote are readily detectable), with no environmental variance component, *i.e.*, heritability of 1.

5 **Phenotype:** The detectable characteristics of a cell or organism, which characteristics are the manifestation of gene expression.

Quantitative Trait Loci (QTL): Genetic loci that contribute, at least in part, certain numerically representable traits that are usually continuously distributed.

Regeneration: The development of a plant from tissue culture.

10 **SSR profile:** A profile of simple sequence repeats used as genetic markers and scored by gel electrophoresis following PCR™ amplification using flanking oligonucleotide primers.

Self-pollination: The transfer of pollen from the anther to the stigma of the same plant.

15 **Tissue Culture:** A composition comprising isolated cells of the same or a different type or a collection of such cells organized into parts of a plant.

Transgene: A genetic sequence which has been introduced into the nuclear or chloroplast genome of a maize plant by a genetic transformation technique.

20 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing
25 from the spirit and scope of the invention.

III. INBRED CORN PLANT LH332

A. Origin and Breeding History

LH332 was developed from an initial cross of HC33 x LH198 by selfing and use of the pedigree system of plant breeding. Yield, stalk quality, root quality, disease
5 tolerance, late plant greenness, late plant intactness, ear retention, pollen shedding ability, silking ability and corn borer tolerance were the criteria used to determine the rows from which ears were selected.

LH198 is a proprietary field corn inbred line of Holden's Foundation Seeds, LLC.
10 PVPA certificate #9200021 was awarded for LH198 on March 31, 1993. U.S. Patent Office No. 5,304,717, issued April 19, 1994, also protects LH198. HC33 is a line developed by Asgrow Seed Co. and is protected by U.S. Patent No. 5,714,671.

LH332 has shown uniformity and stability for all traits. It has been self-pollinated and ear-rowed a sufficient number of generations, with careful attention to uniformity of
15 plant type to ensure homozygosity and phenotypic stability. The line has been increased both by hand (Indiana 2001 and 2002) and sibbed in isolated production fields (Hawaii 2003 and Iowa 2003) with continued observations for uniformity. The originating plant breeder has observed LH332 all four generations it has been increased. The line is uniform, stable and no variant traits have been observed or are anticipated in LH332.

20 Inbred corn plants can be reproduced by planting the seeds of the inbred corn plant LH332, growing the resulting corn plants under self-pollinating or sib-pollinating conditions with adequate isolation using standard techniques well known to an artisan skilled in the agricultural arts. Seeds can be harvested from such a plant using standard, well known procedures.

25 B. Phenotypic Description

In accordance with another aspect of the present invention, there is provided a corn plant having the physiological and morphological characteristics of corn plant LH332. A description of the physiological and morphological characteristics of corn plant LH332 is presented in Table 1.

TABLE 1: Physiological and Morphological Traits for the LH332 Phenotype

TYPE: Dent

REGION WHERE DEVELOPED: Northcentral U.S.

MATURITY:

	<u>Days</u>	<u>Heat</u> <u>Units</u>
From emergence to 50% of plants in silk:	74	1473
From emergence to 50% of plants in pollen	74	1473

$$\text{Heat Units:} = \frac{[\text{Max. Temp. } (\leq 86^{\circ}\text{F.}) + \text{Min. Temp. } (\geq 50^{\circ}\text{F.})] - 50}{2}$$

PLANT:

Plant Height (to tassel tip): 226.6 cm

Ear Height (to base of top ear): 95.3 cm

Length of Top Ear Internode: 12.5 cm

Average Number of Ears per Stalk: 1.0

Anthocyanin of Brace Roots: Dark

LEAF:

Width of Ear Node Leaf: 7.2 cm

Length of Ear Node Leaf: 76.5 cm

Number of leaves above top ear: 5.8

Leaf Angle (from 2nd Leaf above ear at anthesis to Stalk above leaf): 18.0°

Leaf Color: Dark Green- Munsell Code 5 GY $\frac{3}{4}$

Leaf Sheath Pubescence (Rate on scale from 1 =none to 9=like peach fuzz): 3

Marginal Waves (Rate on scale from 1 =none to 9=many): 8

Longitudinal Creases (Rate on scale from 1 =none to 9=many): 4

TASSEL:

Number of Lateral Branches: 6.3

Branch Angle from Central Spike: 30.5°

Tassel Length (from top leaf collar to tassel top): 42.9 cm

Anther Color: Yellow - Munsell Code 2.5 Y 8/10

Glume Color: Purple - Munsell Code 5 RP 5/8

Bar Glumes: Absent

EAR: (Unhusked Data)

Silk Color (3 days after emergence): Green-Yellow - Munsell Code 2.5 GY 8/6

Fresh Husk Color (25 days after 50% silking): Light Green - Munsell Code 2.5 GY 7/6

Dry Husk Color (65 days after 50% silking): Buff - Munsell Code 7.5 YR 7/4

Position of Ear: Upright

Husk Tightness (Rate on scale from 1 =very loose to 9=very tight): 9

Husk Extension: Short (ears exposed)

EAR: (Husked Ear Data)

Ear Length: 11.5 cm

Ear Diameter at mid-point: 41.2 mm

Number of Kernel Rows: 13.2

Kernel Rows: Distinct

Row Alignment: Straight

Shank Length: 8.0 cm

Ear Taper: Average

KERNEL: (Dried)

Kernel Length: 10.6 mm

Kernel Width: 7.8 mm

Kernel Thickness: 4.0 mm

Aleurone Color Pattern: Homozygous

Aleurone Color: White - Munsell Code 2.5Y 8/2

Hard Endosperm Color: Yellow - Munsell Code 2.5Y 7/8

Endosperm Type: Normal Starch

COB:

Cob Diameter at Mid-Point: 22.0 mm

Cob Color: Pink - Munsell code 5 R 6/6

AGRONOMIC TRAITS:

0% Dropped Ears (at 65 days after anthesis)

0% Pre-anthesis Brittle Snapping

0% Pre-anthesis Root Lodging

0% Post-anthesis Root Lodging (at 65 days after anthesis)

**These are typical values. Values may vary due to environment. Other values that are substantially equivalent are also within the scope of the invention.*

5 C. Deposit Information

A representative deposit of 2500 seeds of the inbred corn variety designated LH332 has been made with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA on (_____, ____). Those deposited seeds have been assigned ATCC Accession No. - - - - -. The deposit was made in accordance with the
10 terms and provisions of the Budapest Treaty relating to deposit of microorganisms and was made for a term of at least thirty (30) years and at least five (05) years after the most recent request for the furnishing of a sample of the deposit is received by the depository, or for the effective term of the patent, whichever is longer, and will be replaced if it becomes non-viable during that period.

15

IV. CONVERSIONS OF VARIETY LH332

When the term inbred corn plant is used in the context of the present invention, this also includes any conversions of that inbred. It is understood to those of skill in the art that, using backcrossing, essentially any locus may be introduced into the novel corn
20 variety described herein. The term converted plant as used herein refers to those corn plants which are developed by a plant breeding technique called backcrossing, wherein essentially all of the desired morphological and physiological characteristics of an inbred are recovered in addition to a genetic locus transferred into the inbred *via* the backcrossing technique.

25 Backcrossing methods can be used with the present invention to improve or introduce a trait into the inbred. The term backcrossing as used herein refers to the repeated crossing of a hybrid progeny back to one of the parental corn plants for that

inbred. The parental corn plant which contributes the locus or loci for the desired trait is termed the nonrecurrent or donor parent. This terminology refers to the fact that the nonrecurrent parent is used one time in the backcross protocol and therefore does not recur.

5 The parental corn plant to which the locus or loci from the nonrecurrent parent are transferred is known as the recurrent parent as it is used for several rounds in the backcrossing protocol (Poehlman *et al.*, 1995; Fehr, 1987; Sprague and Dudley, 1988). In a typical backcross protocol, the original inbred of interest (recurrent parent) is crossed to a second inbred (nonrecurrent parent) that carries the genetic locus of interest to be
10 transferred. The resulting progeny from this cross are then crossed again to the recurrent parent and the process is repeated until a corn plant is obtained wherein essentially all of the desired morphological and physiological characteristics of the recurrent parent are recovered in the converted plant, in addition to the transferred locus from the nonrecurrent parent. The backcross process may be accelerated by the use of genetic
15 markers, such as SSR, RFLP, SNP or AFLP markers to identify plants with the greatest genetic complement from the recurrent parent.

 The selection of a suitable recurrent parent is an important step for a successful backcrossing procedure. The goal of a backcross protocol is to add or substitute one or more new traits in the original inbred. To accomplish this, a genetic locus of the
20 recurrent inbred is modified or substituted with the desired locus from the nonrecurrent parent, while retaining essentially all of the rest of the desired genetic, and therefore the desired physiological and morphological constitution of the original inbred. The choice of the particular nonrecurrent parent will depend on the purpose of the backcross; one of the major purposes is to add some commercially desirable, agronomically important trait
25 to the plant. The exact backcrossing protocol will depend on the characteristic or trait being altered to determine an appropriate testing protocol. Although backcrossing methods are simplified when the characteristic being transferred is a dominant allele, a recessive allele may also be transferred. In this instance it may be necessary to introduce a test of the progeny to determine if the desired characteristic has been successfully
30 transferred.

Many traits have been identified that are not regularly selected for in the development of a new inbred but that can be improved by backcrossing techniques. A genetic locus conferring the traits may or may not be transgenic. Examples of such traits known to those of skill in the art include, but are not limited to, male sterility, waxy starch, herbicide resistance, resistance for bacterial, fungal, or viral disease, insect resistance, male fertility, enhanced nutritional quality, industrial usage, yield stability, and yield enhancement. These genes are generally inherited through the nucleus, but may be inherited through the cytoplasm. Some known exceptions to this are genes for male sterility, some of which are inherited cytoplasmically, but still act as a single locus trait. A number of exemplary genetic loci conferring new traits are described in, for example, PCT Application WO 95/06128, the disclosure of which is specifically incorporated herein by reference.

Examples of genes conferring male sterility include those disclosed in U.S. Patent No. 3,861,709, U.S. Patent No. 3,710,511, U.S. Patent No. 4,654,465, U.S. Patent No. 5,625,132, and U.S. Patent No. 4,727,219, each of the disclosures of which are specifically incorporated herein by reference in their entirety. A particularly useful type of male sterility gene is one which can be induced by exposure to a chemical agent, for example, a herbicide (U.S. Patent Ser. No. 08/927,368, filed September 11, 1997, the disclosure of which is specifically incorporated herein by reference in its entirety). Both inducible and non-inducible male sterility genes can increase the efficiency with which hybrids are made, in that they eliminate the need to physically emasculate the corn plant used as a female in a given cross.

Where one desires to employ male-sterility systems with a corn plant in accordance with the invention, it may be beneficial to also utilize one or more male-fertility restorer genes. For example, where cytoplasmic male sterility (CMS) is used, hybrid seed production requires three inbred lines: (1) a cytoplasmically male-sterile line having a CMS cytoplasm; (2) a fertile inbred with normal cytoplasm, which is isogenic with the CMS line for nuclear genes ("maintainer line"); and (3) a distinct, fertile inbred with normal cytoplasm, carrying a fertility restoring gene ("restorer" line). The CMS line is propagated by pollination with the maintainer line, with all of the progeny being male

sterile, as the CMS cytoplasm is derived from the female parent. These male sterile plants can then be efficiently employed as the female parent in hybrid crosses with the restorer line, without the need for physical emasculation of the male reproductive parts of the female parent.

5 The presence of a male-fertility restorer gene results in the production of fully fertile F₁ hybrid progeny. If no restorer gene is present in the male parent, male-sterile hybrids are obtained. Such hybrids are useful where the vegetative tissue of the corn plant is utilized, *e.g.*, for silage, but in most cases, the seeds will be deemed the most valuable portion of the crop, so fertility of the hybrids in these crops must be restored.

10 Therefore, one aspect of the current invention concerns the inbred corn plant LH332 comprising a genetic locus capable of restoring male fertility in an otherwise male-sterile inbred or hybrid plant. Examples of male-sterility genes and corresponding restorers which could be employed with the inbred of the invention are well known to those of skill in the art of plant breeding and are disclosed in, for instance, U.S. Patent No. 5,530,191;

15 U.S. Patent No. 5,689,041; U.S. Patent No. 5,741,684; and U.S. Patent No. 5,684,242, the disclosures of which are each specifically incorporated herein by reference in their entirety.

 Direct selection may be applied where a genetic locus acts as a dominant trait. An example of a dominant trait is the herbicide resistance trait. For this selection process,

20 the progeny of the initial cross are sprayed with the herbicide prior to the backcrossing. The spraying eliminates any plants which do not have the desired herbicide resistance characteristic, and only those plants which have the herbicide resistance gene are used in the subsequent backcross. This process is then repeated for all additional backcross generations.

25 Many useful traits are those which are introduced by genetic transformation techniques. Methods for the genetic transformation of maize are known to those of skill in the art. For example, methods which have been described for the genetic transformation of maize include electroporation (U.S. Patent No. 5,384,253), electrotransformation (U.S. Patent No. 5,371,003), microprojectile bombardment (U.S.

Patent No. 5,550,318; U.S. Patent No. 5,736,369, U.S. Patent No. 5,538,880; and PCT Publication WO 95/06128), *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and E.P. Publication EP672752), direct DNA uptake transformation of protoplasts (Omirulleh *et al.*, 1993) and silicon carbide fiber-mediated transformation
5 (U.S. Patent No. 5,302,532 and U.S. Patent No. 5,464,765).

A type of trait which can be introduced by genetic transformation (U.S. Patent No. 5,554,798) and has particular utility is a gene which confers resistance to the herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS, which is active in the biosynthetic pathway of aromatic amino acids. Inhibition of this enzyme leads to
10 starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived therefrom. Mutants of this enzyme are available which are resistant to glyphosate. For example, U.S. Patent 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance upon organisms having the *Salmonella typhimurium* gene for EPSPS, *aroA*. A mutant EPSPS gene having similar mutations has
15 also been cloned from *Zea mays*. The mutant gene encodes a protein with amino acid changes at residues 102 and 106 (PCT Publication WO 97/04103). When a plant comprises such a gene, a herbicide resistant phenotype results.

Plants having inherited a transgene comprising a mutated EPSPS gene may, therefore, be directly treated with the herbicide glyphosate without the result of
20 significant damage to the plant. This phenotype provides farmers with the benefit of controlling weed growth in a field of plants having the herbicide resistance trait by application of the broad spectrum herbicide glyphosate. For example, one could apply the herbicide ROUNDUP™, a commercial formulation of glyphosate manufactured and sold by the Monsanto Company, over the top in fields where glyphosate resistant corn
25 plants are grown. The herbicide application rates may typically range from 4 ounces of ROUNDUP™ to 256 ounces ROUNDUP™ per acre. More preferably, about 16 ounces to about 64 ounces per acre of ROUNDUP™ may be applied to the field. However, the application rate may be increased or decreased as needed, based on the abundance and / or type of weeds being treated. Additionally, depending on the location of the field and
30 weather conditions, which will influence weed growth and the type of weed infestation, it

may be desirable to conduct further glyphosate treatments. The second glyphosate application will also typically comprise an application rate of about 16 ounces to about 64 ounces of ROUNDUP™ per acre treated. Again, the treatment rate may be adjusted based on field conditions. Such methods of application of herbicides to agricultural crops are well known in the art and are summarized in general in Anderson (1983).

Alternatively, more than one trait may be introgressed into an elite inbred by the method of backcross conversion. A selectable marker gene and a gene encoding a protein which confers a trait of interest may be simultaneously introduced into a maize plant as a result of genetic transformation. Usually one or more introduced genes will integrate into a single chromosome site in the host cell's genome. For example, a selectable marker gene encoding phosphinothricin acetyl transferase (PPT) (*e.g.*, a bar gene) and conferring resistance to the active ingredient in some herbicides by inhibiting glutamine synthetase, and a gene encoding an endotoxin from *Bacillus thuringiensis* (Bt) and conferring resistance to particular classes of insects, *e.g.*, lepidopteran insects, in particular the European Corn Borer, may be simultaneously introduced into a host genome. Furthermore, through the process of backcross conversion more than one transgenic trait may be transferred into an elite inbred.

The waxy characteristic is an example of a recessive trait. In this example, the progeny resulting from the first backcross generation (BC1) must be grown and selfed. A test is then run on the selfed seed from the BC1 plant to determine which BC1 plants carried the recessive gene for the waxy trait. In other recessive traits additional progeny testing, for example growing additional generations such as the BC1S1, may be required to determine which plants carry the recessive gene.

It is understood to those of skill in the art that a single locus of transgenic origin need not be directly transformed into a plant, as techniques for the production of stably transformed corn plants that pass single loci to progeny by Mendelian inheritance is well known in the art. Such single loci may therefore be passed from parent plant to progeny plants by standard plant breeding techniques that are well known in the art.

Many thousands of single locus traits are known to those of skill in the art and may be transferred into the variety described herein using well known techniques. Non-limiting examples of some such single locus traits that may be introduced into a plant of the invention according to well known methods are as follows: the *uidA* gene from *E. Coli* encoding β -glucuronidase (GUS) (cells expressing *uidA* produce a blue color when given the appropriate substrate, Jefferson, R.A. 1987. *Plant Mol. Biol. Rep* 5: 387-405); the *bar* gene from *Streptomyces hygroscopicus* encoding phosphinothricin acetyltransferase (PAT) (cells expressing PAT are resistant to the herbicide Basta, White *et al.*, 1990. *Nucl. Ac. Research* 18: 1062); the *lux* gene from firefly encoding luciferase (cells expressing *lux* emit light under appropriate assay conditions, deWet *et al.*, 1987. *Mol. Cell. Biol.* 7: 725-737); the *dhfr* gene from mouse encoding dihydrofolate reductase (DHFR) (cells expressing *dhfr* are resistant to methotrexate; Eichholtz *et al.*, 1987. *Somatic Cell Mol. Genet.* 13: 67-76); the *neo* gene from *E. coli* encoding aminoglycoside phosphotransferase (APH) (cells expressing *neo* are resistant to the aminoglycoside antibiotics; Beck *et al.*, 1982. *Gene* 19: 327-336); the *amp* gene from *E. Coli* encoding β -lactamase (cells expressing β -lactamase produce a chromogenic compound when given the appropriate substrate; Sutcliffe, J.G. 1978. *Proc. Nat. Acad. Sci. USA* 75: 3737-3741); the *xylE* gene from *Ps. putida* encoding catechol dihydroxygenase (cells expressing *xylE* produce a chromogenic compound when given the appropriate substrate; Zukowsky *et al.*, 1983. *Proc. Nat. Acad. Sci. USA* 80: 1101-1105); the R, C1 and B genes from maize encode proteins that regulate anthocyanin biosynthesis in maize (Goff *et al.*, 1990. *EMBO J.*:2517-2522); the ALS gene from *Zea mays* encoding acetolactate synthase and mutated to confer resistance to sulfonylurea herbicides (cells expressing ALS are resistant to the herbicide; Gleen *et al.*, 1992. *Plant Molecular Biology* 18: 1185-1187); the proteinase inhibitor II gene from potato and tomato (plants expressing the proteinase inhibitor II gene show increased resistance to insects; potato – Graham *et al.*, 1986. *Mol. Cell. Biol.* 2: 1044-1051; tomato – Pearce *et al.*, 1991. *Science* 253:895-898); the *Bt* gene from *Bacillus thuringiensis* berliner 1715 encoding a protein that is toxic to insects (this gene is the coding sequence of *Bt* 884 modified in two regions for improved expression in plants; Vaeck *et al.*, 1987. *Nature*

328: 33-37); the *bxn* gene from *Klebsiella ozaenae* encoding a nitrilase enzyme specific for the herbicide bromoxynil (cells expressing this gene are resistant to the herbicide bromoxynil; Stalker *et al.*, *Science* 242: 419-422, 1988); the WGA-A gene encoding wheat germ agglutinin (expression of the WGA-A gene confers resistance to insects; Smith and Raikhel, 1989. *Plant Mol. Biology* 13: 601-603); the *dapA* gene from *E. coli* encoding dihydrodipicolinate synthase (expression of this gene in plant cells produces increased levels of free lysine; Richaud *et al.*, 1986. *J. Bacteriol.* 166: 297-300); the *Z10* gene encoding a 10kD zein storage protein from maize (expression of this gene in cells alters the quantities of 10kD Zein in the cells; Kirihaara *et al.*, 1988. *Mol. Gen. Genet.* 211: 477-484); the Bt gene cloned from *Bacillus thuringiensis* Kurstaki encoding a protein that is toxic to insects (the gene is the coding sequence of the cry IA(c) gene modified for improved expression in plants - plants expressing this gene are resistant to insects; Höfte and Whiteley, 1989. *Microbiological Reviews.* 53: 242-255); the ALS gene from *Arabidopsis thaliana* encoding a sulfonylurea herbicide resistant acetolactate synthase enzyme (cells expressing this gene are resistant to the herbicide Gleen *et al.*, 1988. *Mol. Gen. Genet.* 211: 266-271); the *deh1* gene from *Pseudomonas putida* encoding a dehalogenase enzyme (cells expressing this gene are resistant to the herbicide Dalapon; Buchanan-Wollaston *et al.*, 1992. *Plant Cell Reports* 11: 627-631); the hygromycin phosphotransferase II gene from *E. coli* (expression of this gene in cells produces resistance to the antibiotic hygromycin; Waldron *et al.*, 1985. *Plant Molecular Biology* 5: 103-108); the *mtlD* gene cloned from *E. coli* (the gene encodes the enzyme mannitol-1-phosphate dehydrogenase; Lee and Saier, 1983. *J. of Bacteriol.* 153:685); the HVA-1 gene encoding a Late Embryogenesis Abundant (LEA) protein (the gene was isolated from barley; Dure *et al.*, *Plant Molecular Biology* 12: 475-486); a short amino acid sequence able to specify nuclear location, (Steifel *et al.*, 1984. *Cell* 39:499-509); a maize cell wall hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation (*Plant Cell* 2:785-793, 1990); plant disease resistance genes such as those activated by specific interaction between the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence (*Avr*) gene in the pathogen (Jones *et al.*, 1994. *Science* 266:7891), the tomato *Cf-9* gene for resistance to *Cladosporium*

(Martin *et al.*, 1993. *Science* 262: 1432), a tomato *Pto* gene for resistance to *Pseudomonas syringae* pv. (Llindinos *et al.*, 1994. *Cell* 78: 1089), *Arabidopsis* RSP2 gene for resistance to *Pseudomonas syringae*; a gene conferring resistance to a pest, such as soybean cyst nematode (PCT Application W096/30517; PCT Application W093/19181), a lectin (Van Damme *et al.*, 1994. *Plant Molec. Biol.* 24:25, a vitamin-binding protein such as avidin (PCT application US93/06487); an enzyme inhibitor, for example, a protease or proteinase inhibitor or an amylase inhibitor (Abe *et al.*, 1987. *J. Biol. Chem.* 262:16793), a nucleotide sequence of rice cysteine proteinase inhibitor (Huub *et al.*, 1993. *Plant Molec. Biol.* 21:985), a nucleotide sequence of encoding tobacco proteinase inhibitor I (Sumitani *et al.*, 1993. *Biosci. Biotech. Biochem.* 57:1243), a nucleotide sequence of *Streptomyces nitrosporeus* α-amylase inhibitor (U. S. Patent No. 5,494,813); an insect-specific hormone or pheromone such as an ecysteroid and juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof (Hammock *et al.*, 1990. *Nature* 344:458); an insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest (Regan, 1994. *J. Biol. Chem.* 269:9); an insect-specific venom produced in nature by a snake, a wasp, etc. (Pang *et al.*, 1992. *Gene* 116:165), an enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic (PCT application WO 93/02197), which discloses the nucleotide sequence of a callase gene; DNA molecules which contain chitinase-encoding sequences (Kramer *et al.*, 1993. *Insect Biochem. Molec. Biol.* 23:691), who teach the nucleotide sequence of a cDNA encoding tobacco hookworm chitinase, and Kawalleck *et al.*, 1993. *Plant Molec. Biol.* 21 :673), who provide the nucleotide sequence; a molecule that stimulates signal transduction (Botella *et al.*, 1994. *Plant Molec. Biol.* 24:757) of nucleotide sequences for mung bean calmodulin cDNA clones, and (Griess *et al.*, 1994. *Plant Physiol.* 104:1467), who provide the nucleotide sequence of a maize calmodulin cDNA clone; a sequence of the parsley ubi4-2 polyubiquitin gene peptide (PCT application W095/16776); a membrane

permease, a channel former or a channel blocker (Jaynes *et al.*, 1993. *Plant Sci* 89:43), of
 heterologous -expression of a cecropin-P, lytic peptide analog to render transgenic
 tobacco plants resistant to *Pseudomonas solanacearum*; a viral-invasive protein or a
 complex toxin derived therefrom, for example, the accumulation of viral coat proteins in
 5 transformed plant cells imparts resistance to viral infection and/or disease development
 effected by the virus from which the coat protein gene is derived, as well as by related
 viruses (Beachy *et al.*, 1990. *Ann. rev. Phytopathol.* 28:451); an insect-specific antibody
 or an immunotoxin derived therefrom (Cf. Taylor *et al.*, 1994. Abstract W97, *Seventh*
Int'l Symposium on Molecular Plant-Microbe Interactions (Edinburgh, Scotland); a virus-
 10 specific antibody (Tavladoraki *et al.*, 1993. *Nature* 366:469), who show that transgenic
 plants expressing recombinant antibody genes are protected from virus attack; a
 developmental-arrestive protein produced in nature by a pathogen or a parasite by
 solubilizing plant cell wall homo-a-I ,4-D-galacturonase (Lamb *et al.*, 1992.
Biotechnology 10:1436); a gene encoding a bean endopolygalacturonase-inhibiting
 15 protein (Toubart *et al.*, 1992. *Plant J.* 2:367); a development-arrestive protein produced in
 nature by a plant (Logemann *et al.*, 1992. *Biotechnology* 10:305); genes that confer
 resistance to a herbicide, for example, a herbicide that inhibits the growing point or
 meristem, such as a mutant ALS and AHAS enzyme (Lee *et al.*, 1988. *EMBO J.* 7:1241;
 Miki *et al.*, 1990. *Theor. Appl. Genet.* 80:449), and pyridinoxy or phenoxy proprionic
 20 acids and cycloshexones (ACCase inhibitor-encoding genes) (U.S. Patent No. 4,940,835,
 Shah, *et al.*), which discloses the nucleotide sequence of a form of EPSP which can
 confer glyphosate resistance, the nucleotide sequence of a phosphinothricin-acetyl-
 transferase gene in European application No. 0 242 246 to Leemans *et al.*, DeGreef *et al.*,
 1989. *Biotechnology* 7:61; genes conferring resistance to phenoxy proprionic acids and
 25 clycloshexones, such as sethoxydim and haloxyfop, including Accl-S1 , Accl-S2 and
 Accl-S3 genes (Marshall *et al.*, 1992. *Theor. Appl. Genet.* 83:4:35); a herbicide that
 inhibits photosynthesis, such as a triazine (*psbA* and *gs+* genes) and a benzonitrile
 (nitrilase gene) (Przibila *et al.*, 1991. *Plant Cell* 3:169); mutant *psbA* genes; nitrilase
 genes (U. S. Patent No. 4,810,648 to Stalker); modified fatty acid metabolism genes, for
 30 example, an antisense gene of stearyl-ACP desaturase to increase stearic acid content of

the plant (Knutzon *et al.*, 1992. *Proc. Natl. Acad. Sci. U.S.A.* 89:2624); a phytase-encoding gene to enhance breakdown of phytate, adding more free phosphate to the transformed plant (Van Hartingsveldt *et al.*, 1993. *Gene* 127:87); an *Aspergillus niger* phytase gene; a gene coding for an enzyme that alters the branching pattern of starch; also
 5 (Shiroza *et al.*, 1988. *J. Bact.* 170:810) (nucleotide sequence of *Streptococcus mutans* fructosyltransferase gene), (Steinmetz *et al.*, 1985. *Mol. Gen. Genet.* 20:220) (nucleotide sequence of *Bacillus subtilis* levansucrase gene), (Pen *et al.*, 1992. *Biotechnology* 10:292) (production of transgenic plants that express *Bacillus licheniformis* amylase), (Elliot *et al.*, 1993. *Plant Molec. Biol.* 21 515) (nucleotide sequences of tomato invertase genes),
 10 Sergaard *et al.*, 1993. *J. Biol. Chem.* 268:22480) (site-directed mutagenesis of barley α-amylase gene), and (Fisher *et al.*, 1993. *Plant Physiol.* 102: 1 045) (maize endosperm starch branching enzyme II).

A. Origin And Breeding History Of An Exemplary Converted Plant

85DGD1 MLms is a conversion of 85DGD1 to cytoplasmic male sterility.
 15 85DGD1 MLms was derived using backcross methods. 85DGD1 (a proprietary inbred of Monsanto Company) was used as the recurrent parent and MLms, a germplasm source carrying ML cytoplasmic sterility, was used as the nonrecurrent parent. The breeding history of the converted inbred 85DGD1 MLms can be summarized as follows:

Hawaii Nurseries Planting Date 04-02-1992	Made up S-O: Female row 585 male row 500
Hawaii Nurseries Planting Date 07-15-1992	S-O was grown and plants were backcrossed times 85DGD1 (rows 444 ' 443)
Hawaii Nurseries Planting Date 11-18-1992	Bulked seed of the BC1 was grown and backcrossed times 85DGD1 (rows V3-27 ' V3-26)
Hawaii Nurseries Planting Date 04-02-1993	Bulked seed of the BC2 was grown and backcrossed times 85DGD1 (rows 37 ' 36)
Hawaii Nurseries Planting Date 07-14-1993	Bulked seed of the BC3 was grown and backcrossed times 85DGD1 (rows 99 ' 98)
Hawaii Nurseries Planting Date 10-28-1993	Bulked seed of BC4 was grown and backcrossed times 85DGD1 (rows KS-63 ' KS-62)

Summer 1994	A single ear of the BC5 was grown and backcrossed times 85DGD1 (MC94-822 ' MC94-822-7)
Winter 1994	Bulked seed of the BC6 was grown and backcrossed times 85DGD1 (3Q-1 ' 3Q-2)
Summer 1995	Seed of the BC7 was bulked and named 85DGD1 MLms.

B. Illustrative Procedures for Production of Converted Plants

As described above, techniques for the production of converted corn plants are well known in the art (see, *e.g.*, Poehlman *et al.*, 1995; Fehr, 1987; Sprague and Dudley, 1988). A non-limiting example of such a procedure one of skill in the art would use for preparation of a conversion of corn plant LH332 is as follows:

- (a) crossing corn plant LH332 to a second (nonrecurrent) corn plant comprising a locus to be converted in corn plant LH332;
- (b) selecting at least a first progeny plant resulting from the crossing and comprising the locus;
- (c) crossing the selected progeny to corn plant LH332; and
- (d) repeating steps (b) and (c) until a plant of variety LH332 is obtained comprising the locus.

Following these steps, essentially any locus may be introduced into corn plant LH332. The locus can be introduced without regard to whether the locus confers any given trait. For example, molecular techniques allow introduction of any given locus, without the need for phenotypic screening of progeny during the backcrossing steps.

PCR and Southern hybridization are two examples of molecular techniques that may be used for confirmation of the presence of a given locus and thus conversion of that locus. The techniques are carried out as follows: Seeds of progeny plants are grown and DNA isolated from leaf tissue (see Sambrook *et al.*, 1989; Shure *et al.* 1983). Approximately one gram of leaf tissue is lyophilized overnight in 15 ml polypropylene tubes. Freeze-dried tissue is ground to a power in the tube using a glass rod. Powdered

tissue is mixed thoroughly with 3 ml extraction buffer (7.0 urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 3 ml phenol/chloroform. The aqueous phase is separated by centrifugation, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate pH 5.2, and an equal
5 volume of isopropanol. The precipitate is washed with 75% ethanol and resuspended in 100-500 µl TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0). The DNA may then be screened as desired for presence of the locus.

For PCR, two hundred to 1000 ng genomic DNA from the progeny plant being screened is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂,
10 50 mM KCl, 0.1 mg/ml gelatin, 200µM each dATP, dCTP, dGTP, dTTP, 20% glycerol, 2.5 units Taq DNA polymerase and 0.5µM each of forward and reverse DNA primers that span a segment of the locus being converted. The reaction is run in a thermal cycling machine 3 minutes at 94 C, 39 repeats of the cycle 1 minute at 94 C, 1 minute at 50 C, 30 seconds at 72 C, followed by 5 minutes at 72 C. Twenty µl of each reaction mix is run on
15 a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours. The amplified fragment is detected using an agarose gel. Detection of an amplified fragment corresponding to the segment of the locus spanned by the primers indicates the presence of the locus.

For Southern analysis, plant DNA is restricted, separated in an agarose gel and
20 transferred to a Nylon filter in 10X SCP (20 SCP: 2M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA) according to standard methods (Southern, 1975). Locus DNA or RNA sequences are labeled, for example, radioactively with ³²P by random priming (Feinberg & Vogelstein, 1983). Filters are prehybridized in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml denatured salmon sperm DNA. The labeled probe
25 is denatured, hybridized to the filter and washed in 2X SCP, 1% SDS at 65° for 30 minutes and visualized by autoradiography using Kodak XAR5 film. Presence of the locus is indicated by detection of restriction fragments of the appropriate size.

VI. TISSUE CULTURES AND *IN VITRO* REGENERATION OF CORN PLANTS

A further aspect of the invention relates to tissue cultures of the corn plant designated LH332. As used herein, the term "tissue culture" indicates a composition comprising isolated cells of the same or a different type or a collection of such cells organized into parts of a plant. Exemplary types of tissue cultures are protoplasts, calli and plant cells that are intact in plants or parts of plants, such as embryos, pollen, flowers, kernels, ears, cobs, leaves, husks, stalks, roots, root tips, anthers, silk, and the like. In a preferred embodiment, the tissue culture comprises embryos, protoplasts, meristematic cells, pollen, leaves or anthers derived from immature tissues of these plant parts. Means for preparing and maintaining plant tissue cultures are well known in the art (U.S. Patent No. 5,538,880; and U.S. Patent No. 5,550,318, each incorporated herein by reference in their entirety). By way of example, a tissue culture comprising organs such as tassels or anthers has been used to produce regenerated plants (U.S. Patent No. 5,445,961 and U.S. Patent No. 5,322,789; the disclosures of which are incorporated herein by reference).

One type of tissue culture is tassel/anther culture. Tassels contain anthers which in turn enclose microspores. Microspores develop into pollen. For anther/microspore culture, if tassels are the plant composition, they are preferably selected at a stage when the microspores are uninucleate, that is, include only one, rather than 2 or 3 nuclei. Methods to determine the correct stage are well known to those skilled in the art and include mitramycin fluorescent staining (Pace *et al.*, 1987), trypan blue (preferred) and acetocarmine squashing. The mid-uninucleate microspore stage has been found to be the developmental stage most responsive to the subsequent methods disclosed to ultimately produce plants.

Although microspore-containing plant organs such as tassels can generally be pretreated at any cold temperature below about 25°C, a range of 4 to 25°C is preferred, and a range of 8 to 14°C is particularly preferred. Although other temperatures yield embryoids and regenerated plants, cold temperatures produce optimum response rates compared to pretreatment at temperatures outside the preferred range. Response rate is measured as either the number of embryoids or the number of regenerated plants per number of microspores initiated in culture. Exemplary methods of microspore culture are

disclosed in, for example, U.S. Patent No. 5,322,789 and U.S. Patent No 5,445,961, the disclosures of which are specifically incorporated herein by reference.

Although not required, when tassels are employed as the plant organ, it is generally preferred to sterilize their surface. Following surface sterilization of the tassels, for example, with a solution of calcium hypochloride, the anthers are removed from about 5 70 to 150 spikelets (small portions of the tassels) and placed in a preculture or pretreatment medium. Larger or smaller amounts can be used depending on the number of anthers.

When one elects to employ tassels directly, tassels are preferably pretreated at a cold temperature for a predefined time, preferably at 10°C for about 4 days. After 10 pretreatment of a whole tassel at a cold temperature, dissected anthers are further pretreated in an environment that diverts microspores from their developmental pathway. The function of the preculture medium is to switch the developmental program from one of pollen development to that of embryoid/callus development. An embodiment of such an environment in the form of a preculture medium includes a sugar alcohol, for example 15 mannitol or sorbitol, inositol or the like. An exemplary synergistic combination is the use of mannitol at a temperature of about 10°C for a period ranging from about 10 to 14 days. In a preferred embodiment, 3 ml of 0.3 M mannitol combined with 50 mg/l of ascorbic acid, silver nitrate, and colchicine is used for incubation of anthers at 10°C for between 20 10 and 14 days. Another embodiment is to substitute sorbitol for mannitol. The colchicine produces chromosome doubling at this early stage. The chromosome doubling agent is preferably only present at the preculture stage.

It is believed that the mannitol or other similar carbon structure or environmental stress induces starvation and functions to force microspores to focus their energies on 25 entering developmental stages. The cells are unable to use, for example, mannitol as a carbon source at this stage. It is believed that these treatments confuse the cells causing them to develop as embryoids and plants from microspores. Dramatic increases in development from these haploid cells, as high as 25 embryoids in 10^4 microspores, have resulted from using these methods.

In embodiments where microspores are obtained from anthers, microspores can be released from the anthers into an isolation medium following the mannitol preculture step. One method of release is by disruption of the anthers, for example, by chopping the anthers into pieces with a sharp instrument, such as a razor blade, scalpel, or Waring
5 blender. The resulting mixture of released microspores, anther fragments, and isolation medium are then passed through a filter to separate microspores from anther wall fragments. An embodiment of a filter is a mesh, more specifically, a nylon mesh of about 112 mm pore size. The filtrate which results from filtering the microspore-containing solution is preferably relatively free of anther fragments, cell walls, and other debris.

10 Isolation of microspores can be accomplished at a temperature below about 25°C and preferably, at a temperature of less than about 15°C. Preferably, the isolation media, dispersing tool (*e.g.*, razor blade), funnels, centrifuge tubes, and dispersing container (*e.g.*, petri dish) are all maintained at the reduced temperature during isolation. The use of a precooled dispersing tool to isolate maize microspores has been reported (Gaillard *et*
15 *al.*, 1991).

To isolate microspores, an isolation media is preferred. An isolation media is used to separate microspores from the anther walls while maintaining their viability and embryogenic potential. An illustrative embodiment of an isolation media includes a 6% sucrose or maltose solution combined with an antioxidant such as 50 mg/l of ascorbic
20 acid, 0.1 mg/l biotin, and 400 mg/l of proline, combined with 10 mg/l of nicotinic acid and 0.5 mg/l AgNO₃. In another embodiment, the biotin and proline are omitted.

An isolation media preferably has a higher antioxidant level where it is used to isolate microspores from a donor plant (a plant from which a plant composition containing a microspore is obtained) that is field grown in contrast to greenhouse grown.
25 A preferred level of ascorbic acid in an isolation medium is from about 50 mg/l to about 125 mg/l and, more preferably, from about 50 mg/l to about 100 mg/l.

One can find particular benefit in employing a support for the microspores during culturing and subculturing. Any support that maintains the cells near the surface can be used. An illustrative embodiment of a solid support is a TRANSWELL® culture dish.

Another embodiment of a solid support for development of the microspores is a bilayer plate wherein liquid media is on top of a solid base. Other embodiments include a mesh or a millipore filter. Preferably, a solid support is a nylon mesh in the shape of a raft. A raft is defined as an approximately circular support material which is capable of floating slightly above the bottom of a tissue culture vessel, for example, a petri dish, of about a 60 or 100 mm size, although any other laboratory tissue culture vessel will suffice. In an illustrative embodiment, a raft is about 55 mm in diameter.

Culturing isolated microspores on a solid support, for example, on a 10 mm pore nylon raft floating on 2.2 ml of medium in a 60 mm petri dish, prevents microspores from sinking into the liquid medium and thus avoiding low oxygen tension. These types of cell supports enable the serial transfer of the nylon raft with its associated microspore/embryoids ultimately to full strength medium containing activated charcoal and solidified with, for example, GELRITE™ (solidifying agent).

The liquid medium passes through the mesh while the microspores are retained and supported at the medium-air interface. The surface tension of the liquid medium in the petri dish causes the raft to float. The liquid is able to pass through the mesh; consequently, the microspores stay on top. The mesh remains on top of the total volume of liquid medium.

The culture vessels can be further defined as either (1) a bilayer 60 mm petri plate wherein the bottom 2 ml of medium are solidified with 0.7% agarose overlaid with 1 mm of liquid containing the microspores; (2) a nylon mesh raft wherein a wafer of nylon is floated on 1.2 ml of medium and 1 ml of isolated microspores is pipetted on top; or (3) TRANSWELL® plates wherein isolated microspores are pipetted onto membrane inserts which support the microspores at the surface of 2 ml of medium.

After the microspores have been isolated, they can be cultured in a low strength anther culture medium until about the 50 cell stage when they are subcultured onto an embryoid/callus maturation medium. Medium is defined at this stage as any combination of nutrients that permit the microspores to develop into embryoids or callus. Many examples of suitable embryoid/callus promoting media are well known to those skilled in

the art. These media will typically comprise mineral salts, a carbon source, vitamins, and growth regulators. A solidifying agent is optional. A preferred embodiment of such a media is referred to as "D medium," which typically includes 6N1 salts, AgNO₃ and sucrose or maltose.

5 In an illustrative embodiment, 1 ml of isolated microspores are pipetted onto a 10 mm nylon raft and the raft is floated on 1.2 ml of medium "D," containing sucrose or preferably maltose. Both calli and embryoids can develop. Calli are undifferentiated aggregates of cells. Type I is a relatively compact, organized, and slow growing callus. Type II is a soft, friable, and fast-growing one. Embryoids are aggregates exhibiting
10 some embryo-like structures. The embryoids are preferred for subsequent steps to regenerating plants. Culture medium "D" is an embodiment of medium that follows the isolation medium and replaces it. Medium "D" promotes growth to an embryoid/callus. This medium comprises 6N1 salts at 1/8 the strength of a basic stock solution (major components) and minor components, plus 12% sucrose, or preferably 12% maltose, 0.1
15 mg/l B1, 0.5 mg/l nicotinic acid, 400 mg/l proline and 0.5 mg/l silver nitrate. Silver nitrate is believed to act as an inhibitor to the action of ethylene. Multi-cellular structures of approximately 50 cells each generally arise during a period of 12 days to 3 weeks. Serial transfer after a two week incubation period is preferred.

After the petri dish has been incubated for an appropriate period of time,
20 preferably two weeks in the dark at a predefined temperature, a raft bearing the dividing microspores is transferred serially to solid based media which promote embryo maturation. In an illustrative embodiment, the incubation temperature is 30°C and the mesh raft supporting the embryoids is transferred to a 100 mm petri dish containing the 6N1-TGR-4P medium, an "anther culture medium." This medium contains 6N1 salts,
25 supplemented with 0.1 mg/l TIBA, 12% sugar (sucrose, maltose, or a combination thereof), 0.5% activated charcoal, 400 mg/l proline, 0.5 mg/l B, 0.5 mg/l nicotinic acid, and 0.2 percent GELRITE™ (solidifying agent) and is capable of promoting the maturation of the embryoids. Higher quality embryoids, that is, embryoids which exhibit more organized development, such as better shoot meristem formation without
30 precocious germination, were typically obtained with the transfer to full strength medium

compared to those resulting from continuous culture using only, for example, the isolated microspore culture (IMC) Medium "D." The maturation process permits the pollen embryoids to develop further in route toward the eventual regeneration of plants. Serial transfer occurs to full strength solidified 6N1 medium using either the nylon raft, the
5 TRANSWELL® membrane, or bilayer plates, each one requiring the movement of developing embryoids to permit further development into physiologically more mature structures. In an especially preferred embodiment, microspores are isolated in an isolation media comprising about 6% maltose, cultured for about two weeks in an embryoid/calli induction medium comprising about 12% maltose and then transferred to a
10 solid medium comprising about 12% sucrose.

At the point of transfer of the raft, after about two weeks of incubation, embryoids exist on a nylon support. The purpose of transferring the raft with the embryoids to a solidified medium after the incubation is to facilitate embryo maturation. Mature embryoids at this point are selected by visual inspection indicated by zygotic embryo-like
15 dimensions and structures and are transferred to the shoot initiation medium. It is preferred that shoots develop before roots, or that shoots and roots develop concurrently. If roots develop before shoots, plant regeneration can be impaired. To produce solidified media, the bottom of a petri dish of approximately 100 mm is covered with about 30 ml of 0.2% GELRITE™ solidified medium. A sequence of regeneration media are used for
20 whole plant formation from the embryoids.

During the regeneration process, individual embryoids are induced to form plantlets. The number of different media in the sequence can vary depending on the specific protocol used. Finally, a rooting medium is used as a prelude to transplanting to soil. When plantlets reach a height of about 5 cm, they are then transferred to pots for
25 further growth into flowering plants in a greenhouse by methods well known to those skilled in the art.

Plants have been produced from isolated microspore cultures by the methods disclosed herein, including self-pollinated plants. The rate of embryoid induction was much higher with the synergistic preculture treatment consisting of a combination of

stress factors, including a carbon source which can be capable of inducing starvation, a cold temperature, and colchicine, than has previously been reported. An illustrative embodiment of the synergistic combination of treatments leading to the dramatically improved response rate compared to prior methods, is a temperature of about 10°C, mannitol as a carbon source, and 0.05% colchicine.

The inclusion of ascorbic acid, an anti-oxidant, in the isolation medium is preferred for maintaining good microspore viability. However, there seems to be no advantage to including mineral salts in the isolation medium. The osmotic potential of the isolation medium was maintained optimally with about 6% sucrose, although a range of 2% to 12% is within the scope of this invention.

In an embodiment of the embryoid/callus organizing media, mineral salts concentration in IMC Culture Media "D" is (1/8×), the concentration which is used also in anther culture medium. The 6N1 salts major components have been modified to remove ammonium nitrogen. Osmotic potential in the culture medium is maintained with about 12% sucrose and about 400 mg/l proline. Silver nitrate (0.5 mg/l) was included in the medium to modify ethylene activity. The preculture media is further characterized by having a pH of about 5.7 to 6.0. Silver nitrate and vitamins do not appear to be crucial to this medium but do improve the efficiency of the response.

Examples of processes of tissue culturing and regeneration of corn are described in, for example, European Patent Application 0 160 390, Green and Rhodes (1982) and Duncan *et al.* (1985), Songstad *et al.* (1988), Rao *et al.* (1986), Conger *et al.* (1987), PCT Application WO 95/06128, Armstrong and Green, 1985; Gordon-Kamm *et al.*, 1990, and U.S. Patent No. 5,736,369.

VII. PROCESSES OF PREPARING CORN PLANTS AND THE CORN PLANTS PRODUCED BY SUCH CROSSES

The present invention provides processes of preparing novel corn plants and corn plants produced by such processes. In accordance with such a process, a first parent corn plant is crossed with a second parent corn plant wherein at least one of the first and

second corn plants is the inbred corn plant LH332. One application of the process is in the production of F₁ hybrid plants. Another important aspect of this process is that it can be used for the development of novel inbred lines. For example, the inbred corn plant LH332 could be crossed to any second plant, and the resulting hybrid progeny each selfed
5 for about 5 to 7 or more generations, thereby providing a large number of distinct, pure-breeding inbred lines. These inbred lines could then be crossed with other inbred or non-inbred lines and the resulting hybrid progeny analyzed for beneficial characteristics. In this way, novel inbred lines conferring desirable characteristics could be identified.

Corn plants (*Zea mays L.*) can be crossed by either natural or mechanical
10 techniques. Natural pollination occurs in corn when wind blows pollen from the tassels to the silks that protrude from the tops of the recipient ears. Mechanical pollination can be effected either by controlling the types of pollen that can blow onto the silks or by pollinating by hand. In one embodiment, crossing comprises the steps of:

- 15 (a) planting in pollinating proximity seeds of a first and a second parent corn plant, and preferably, seeds of a first inbred corn plant and a second, distinct inbred corn plant;
- (b) cultivating or growing the seeds of the first and second parent corn plants into plants that bear flowers;
- 20 (c) emasculating flowers of either the first or second parent corn plant, *i.e.*, treating the flowers so as to prevent pollen production, or alternatively, using as the female parent a male sterile plant, thereby providing an emasculated parent corn plant;
- (d) allowing natural cross-pollination to occur between the first and second parent corn plants;
- 25 (e) harvesting seeds produced on the emasculated parent corn plant; and, where desired,
- (f) growing the harvested seed into a corn plant, preferably, a hybrid corn plant.

Parental plants are typically planted in pollinating proximity to each other by planting the parental plants in alternating rows, in blocks or in any other convenient planting pattern. Where the parental plants differ in timing of sexual maturity, it may be desired to plant the slower maturing plant first, thereby ensuring the availability of pollen from the male parent during the time at which silks on the female parent are receptive to pollen. Plants of both parental parents are cultivated and allowed to grow until the time of flowering. Advantageously, during this growth stage, plants are in general treated with fertilizer and/or other agricultural chemicals as considered appropriate by the grower.

At the time of flowering, in the event that plant LH332 is employed as the male parent, the tassels of the other parental plant are removed from all plants employed as the female parental plant to avoid self-pollination. The detasseling can be achieved manually but also can be done by machine, if desired. Alternatively, when the female parent corn plant comprises a cytoplasmic or nuclear gene conferring male sterility, detasseling may not be required. Additionally, a chemical gametocide may be used to sterilize the male flowers of the female plant. In this case, the parent plants used as the male may either not be treated with the chemical agent or may comprise a genetic factor which causes resistance to the emasculating effects of the chemical agent. Gametocides affect processes or cells involved in the development, maturation or release of pollen. Plants treated with such gametocides are rendered male sterile, but typically remain female fertile. The use of chemical gametocides is described, for example, in U.S. Patent No. 4,936,904, the disclosure of which is specifically incorporated herein by reference in its entirety. Furthermore, the use of Roundup herbicide in combination with glyphosate tolerant maize plants to produce male sterile corn plants is disclosed in U.S. Patent Application Serial No. 08/927,368 and PCT Publication WO 98/44140.

Following emasculation, the plants are then typically allowed to continue to grow and natural cross-pollination occurs as a result of the action of wind, which is normal in the pollination of grasses, including corn. As a result of the emasculation of the female parent plant, all the pollen from the male parent plant is available for pollination because tassels, and thereby pollen bearing flowering parts, have been previously removed from all plants of the inbred plant being used as the female in the hybridization. Of course,

during this hybridization procedure, the parental varieties are grown such that they are isolated from other corn fields to minimize or prevent any accidental contamination of pollen from foreign sources. These isolation techniques are well within the skill of those skilled in this art.

5 Both parental inbred plants of corn may be allowed to continue to grow until maturity or the male rows may be destroyed after flowering is complete. Only the ears from the female inbred parental plants are harvested to obtain seeds of a novel F₁ hybrid. The novel F₁ hybrid seed produced can then be planted in a subsequent growing season in commercial fields or, alternatively, advanced in breeding protocols for purposes of
10 developing novel inbred lines.

 Alternatively, in another embodiment of the invention, both first and second parent corn plants can be from variety LH332. Thus, any corn plant produced using corn plant LH332 forms a part of the invention. As used herein, crossing can mean selfing, backcrossing, crossing to another or the same inbred, crossing to populations, and the
15 like. All corn plants produced using the inbred corn plant LH332 as a parent are, therefore, within the scope of this invention.

A. F₁ Hybrid Corn Plant and Seed Production

 One beneficial use of the instant corn variety is in the production of hybrid seed. Any time the inbred corn plant LH332 is crossed with another, different, corn inbred, a
20 first generation (F₁) corn hybrid plant is produced. As such, an F₁ hybrid corn plant can be produced by crossing LH332 with any second inbred maize plant. Essentially any other corn plant can be used to produce a hybrid corn plant having corn plant LH332 as one parent. All that is required is that the second plant be fertile, which corn plants naturally are, and that the plant is not corn variety LH332.

25 The goal of the process of producing an F₁ hybrid is to manipulate the genetic complement of corn to generate new combinations of genes which interact to yield new or improved traits (phenotypic characteristics). A process of producing an F₁ hybrid typically begins with the production of one or more inbred plants. Those plants are

produced by repeated crossing of ancestrally related corn plants to try to combine certain genes within the inbred plants.

Corn has a diploid phase which means two conditions of a gene (two alleles) occupy each locus (position on a chromosome). If the alleles are the same at a locus, there is said to be homozygosity. If they are different, there is said to be heterozygosity. In a completely inbred plant, all loci are homozygous. Because many loci when homozygous are deleterious to the plant, in particular leading to reduced vigor, less kernels, weak and/or poor growth, production of inbred plants is an unpredictable and arduous process. Under some conditions, heterozygous advantage at some loci effectively bars perpetuation of homozygosity.

A single cross hybrid corn variety is the cross of two inbred plants, each of which has a genotype which complements the genotype of the other. Typically, F_1 hybrids are more vigorous than their inbred parents. This hybrid vigor, or heterosis, is manifested in many polygenic traits, including markedly improved yields, better stalks, better roots, better uniformity and better insect and disease resistance. In the development of hybrids only the F_1 hybrid plants are typically sought. An F_1 single cross hybrid is produced when two inbred plants are crossed. A double cross hybrid is produced from four inbred plants crossed in pairs ($A \times B$ and $C \times D$) and then the two F_1 hybrids are crossed again $(A \times B) \times (C \times D)$.

Thousands of corn varieties are known to those of skill in the art, any one of which could be crossed with corn plant LH332 to produce a hybrid plant. For example, the U.S. Patent & Trademark has issued more than 300 utility patents for corn varieties. The Maize Genetics Cooperation Stock Center, which is supported by the U.S. Department of Agriculture, has a total collection approaching 80,000 individually pedigreed samples ([//w3.ag.uiuc.edu/maize-coop/mgc-info.html](http://w3.ag.uiuc.edu/maize-coop/mgc-info.html)).

Therefore, any F_1 hybrid corn plant or corn seed which is produced with LH332 as a parent is part of the present invention. Examples of such F_1 hybrids which have been produced with LH332 as a parent are given in Table 2. In such hybrid crosses the inbred corn plant LH332 can serve as either the maternal or paternal plant.

B. Development of Corn Varieties Using LH332

The development of new varieties using one or more starting varieties is well known in the art. In accordance with the invention, novel varieties may be created by crossing corn variety LH332 followed by multiple generations of breeding according to such well known methods. New varieties may be created by crossing corn variety LH332 with any second plant. In selecting such a second plant to cross for the purpose of developing novel inbred lines, it may be desired to choose those plants which either themselves exhibit one or more selected desirable characteristics or which exhibit the desired characteristic(s) when in hybrid combination. Examples of potentially desired characteristics include greater yield, better stalks, better roots, resistance to insecticides, herbicides, pests, and disease, tolerance to heat and drought, reduced time to crop maturity, better agronomic quality, higher nutritional value, and uniformity in germination times, stand establishment, growth rate, maturity, and fruit size.

Once initial crosses have been made with corn variety LH332, inbreeding takes place to produce new inbred varieties. Inbreeding requires manipulation by human breeders. Even in the extremely unlikely event inbreeding rather than crossbreeding occurred in natural corn, achievement of complete inbreeding cannot be expected in nature due to well known deleterious effects of homozygosity and the large number of generations the plant would have to breed in isolation. The reason for the breeder to create inbred plants is to have a known reservoir of genes whose gametic transmission is predictable.

The pedigree breeding method involves crossing two genotypes. Each genotype can have one or more desirable characteristics lacking in the other; or, each genotype can complement the other. If the two original parental genotypes do not provide all of the desired characteristics, other genotypes can be included in the breeding population. Superior plants that are the products of these crosses are selfed and selected in successive generations. Each succeeding generation becomes more homogeneous as a result of self-pollination and selection. Typically, this method of breeding involves five or more

generations of selfing and selection: $S_1 \rightarrow S_2$; $S_2 \rightarrow S_3$; $S_3 \rightarrow S_4$; $S_4 \rightarrow S_5$, *etc.* After at least five generations, the inbred plant is considered genetically pure.

Backcrossing can also be used to improve an inbred plant. Backcrossing transfers a specific desirable trait from one inbred or non-inbred source to an inbred that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate locus or loci for the trait in question. The progeny of this cross are then mated back to the superior recurrent parent (A) followed by selection in the resultant progeny for the desired trait to be transferred from the non-recurrent parent. After five or more backcross generations with selection for the desired trait, the progeny are heterozygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other loci. The last backcross generation would be selfed to give pure breeding progeny for the trait being transferred.

The development of a hybrid corn variety involves three steps: (1) the selection of plants from various germplasm pools; (2) the selfing of the selected plants for several generations to produce a series of inbred plants, which, although different from each other, each breed true and are highly uniform; and (3) crossing the selected inbred plants with unrelated inbred plants to produce the hybrid progeny (F_1). During the inbreeding process in corn, the vigor of the plants decreases. Vigor is restored when two unrelated inbred plants are crossed to produce the hybrid progeny (F_1). An important consequence of the homozygosity and homogeneity of the inbred plants is that the hybrid between any two inbreds is always the same. Once the inbreds that give a superior hybrid have been identified, hybrid seed can be reproduced indefinitely as long as the homogeneity of the inbred parents is maintained. Conversely, much of the hybrid vigor exhibited by F_1 hybrids is lost in the next generation (F_2). Consequently, seed from hybrid varieties is not used for planting stock. It is not generally beneficial for farmers to save seed of F_1 hybrids. Rather, farmers purchase F_1 hybrid seed for planting every year.

The development of inbred plants generally requires at least about 5 to 7 generations of selfing. Inbred plants are then cross-bred in an attempt to develop

improved F₁ hybrids. Hybrids are then screened and evaluated in small scale field trials. Typically, about 10 to 15 phenotypic traits, selected for their potential commercial value, are measured. A selection index of the most commercially important traits is used to help evaluate hybrids. Field trials are used over several years for the progressive elimination of hybrids based on detailed evaluation of their phenotype. Eventually, strip trials are conducted to formally compare the experimental hybrids being developed with other hybrids, some of which were previously developed and generally are commercially successful. That is, comparisons of experimental hybrids are made to competitive hybrids to determine if there was any advantage to further development of the experimental hybrids. Examples of such comparisons are presented hereinbelow. After testing is complete, determinations may be made whether commercial development should proceed for a given hybrid.

C. F₁ Hybrid Comparisons

As mentioned above, hybrids are progressively eliminated following detailed evaluations of their phenotype, including formal comparisons with other commercially successful hybrids. Strip trials are used to compare the phenotypes of hybrids grown in as many environments as possible. They are performed in many environments to assess overall performance of the new hybrids and to select optimum growing conditions. Because the corn is grown in close proximity, environmental factors that affect gene expression, such as moisture, temperature, sunlight, and pests, are minimized. For a decision to be made to commercialize a hybrid, it is not necessary that the hybrid be better than all other hybrids. Rather, significant improvements must be shown in at least some traits that would create improvements in some niches.

Examples of such comparative data are set forth hereinbelow in Table 2, which presents a comparison of performance data for a hybrid made with LH332 as one parent, versus selected hybrids of commercial value.

TABLE 2: Comparative Data for a Hybrid Having LH332 as One Inbred Parent

Pedigree	Mean Yield	% M	Y/M	% Stalk	% Root	% Drop	Plant Height (cm)	Ear Height (cm)	Test Weight
LH332 X LH287	197	18.2	10.81	6	6	0	112	43	56.0
LH198BT1.2 X LH287	-10	-0.8	-0.07	2	-3	0	-3	1	-0.1
LH244 X LH185	8	-0.6	0.74	1	3	0	-4	-1	0.2
HC33 X LH287	6	-0.4	0.58	1	0	0	0	6	0.3
LH332 X LH273	197	17.8	11.10	5	3	0	119	46	56.0
LH198BT1.2 X LH273	-10	-1.7	0.45	2	-3	0	6	1	-0.2
HC33 X LH279	1	-1.7	1.04	1	-2	0	6	3	0.3
HC60 X LH279	-2	-1.0	0.46	0	0	0	9	5	-1.2
LH332 X LH172	212	20.4	10.40	2	2	0	97	38	56.3
HC33 X LH172	11	0.3	0.39	0	1	0	2	1	0.2
HC33 X LH320	8	0.7	0.04	0	0	0	1	2	0.5
HC33 X LH277	9	1.0	-0.08	1	0	0	0	1	0.9

VIII. GENETIC COMPLEMENTS

The present invention provides a genetic complement of the inbred corn plant variety designated LH332. Further provided by the invention is a hybrid genetic complement, wherein the complement is formed by the combination of a haploid genetic complement from LH332 and another haploid genetic complement. Means for determining such a genetic complement are well-known in the art.

As used herein, the phrase “genetic complement” means an aggregate of nucleotide sequences, the expression of which defines the phenotype of a corn plant or a cell or tissue of that plant. By way of example, a corn plant is genotyped to determine a representative sample of the inherited markers it possesses. Markers are alleles at a single locus. They are preferably inherited in codominant fashion so that the presence of both alleles at a diploid locus is readily detectable, and they are free of environmental variation, *i.e.*, their heritability is 1. This genotyping is preferably performed on at least one generation of the descendant plant for which the numerical value of the quantitative trait or traits of interest are also determined. The array of single locus genotypes is expressed as a profile of marker alleles, two at each locus. The marker allelic composition of each locus can be either homozygous or heterozygous. Homozygosity is a condition where both alleles at a locus are characterized by the same nucleotide sequence or size of a repeated sequence. Heterozygosity refers to different conditions of the gene at a locus. A preferred type of genetic marker for use with the invention is simple sequence repeats (SSRs), although potentially any other type of genetic marker could be used, for example, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), and isozymes.

A genetic marker profile of an inbred may be predictive of the agronomic traits of a hybrid produced using that inbred. For example, if an inbred of known genetic marker profile and phenotype is crossed with a second inbred of known genetic marker profile and phenotype it is possible to predict the phenotype of the F₁ hybrid based on the combined genetic marker profiles of the parent inbreds. Methods for prediction of hybrid performance from genetic marker data is disclosed in U.S. Patent No. 5,492,547, the

disclosure of which is specifically incorporated herein by reference in its entirety. Such predictions may be made using any suitable genetic marker, for example, SSRs, RFLPs, AFLPs, SNPs, or isozymes.

SSRs are genetic markers based on polymorphisms in repeated nucleotide sequences, such as microsatellites. A marker system based on SSRs can be highly informative in linkage analysis relative to other marker systems in that multiple alleles may be present. Another advantage of this type of marker is that, through use of flanking primers, detection of SSRs can be achieved, for example, by the polymerase chain reaction (PCRTM), thereby eliminating the need for labor-intensive Southern hybridization. The PCRTM detection is done by use of two oligonucleotide primers flanking the polymorphic segment of repetitive DNA. Repeated cycles of heat denaturation of the DNA followed by annealing of the primers to their complementary sequences at low temperatures, and extension of the annealed primers with DNA polymerase, comprise the major part of the methodology. Following amplification, markers can be scored by gel electrophoresis of the amplification products. Scoring of marker genotype is based on the size (number of base pairs) of the amplified segment.

Means for performing genetic analyses using SSR polymorphisms are well known in the art. The SSR analyses reported herein were conducted by Celera AgGen in Davis, CA. This service is available to the public on a contractual basis. This analysis was carried out by amplification of simple repeats followed by detection of marker genotypes using gel electrophoresis. Markers were scored based on the size of the amplified fragment.

The SSR genetic marker profile of the parental inbreds and exemplary resultant hybrid described herein were determined. Because an inbred is essentially homozygous at all relevant loci, an inbred should, in almost all cases, have only one allele at each locus. In contrast, a diploid genetic marker profile of a hybrid should be the sum of those parents, *e.g.*, if one inbred parent had the allele 168 (base pairs) at a particular locus, and the other inbred parent had 172, the hybrid is 168.172 by inference. Subsequent generations of progeny produced by selection and breeding are expected to be of genotype

168, 172, or 168.172 for that locus position. When the F₁ plant is used to produce an inbred, the locus should be either 168 or 172 for that position. Surprisingly, it has been observed that in certain instances, novel SSR genotypes arise during the breeding process.

Another aspect of this invention is a plant genetic complement characterized by a genetic isozyme typing profile. Isozymes are forms of proteins that are distinguishable, for example, on starch gel electrophoresis, usually by charge and/or molecular weight. The techniques and nomenclature for isozyme analysis are described in, for example, Stuber *et al.* (1988), which is incorporated by reference.

A standard set of loci can be used as a reference set. Comparative analysis of these loci is used to compare the purity of hybrid seeds, to assess the increased variability in hybrids compared to inbreds, and to determine the identity of seeds, plants, and plant parts. In this respect, an isozyme reference set can be used to develop genotypic "fingerprints."

The present invention also provides a hybrid genetic complement formed by the combination of a haploid genetic complement of the corn plant LH332 with a haploid genetic complement of a second corn plant. Means for combining a haploid genetic complement from the foregoing inbred with another haploid genetic complement can comprise any method for producing a hybrid plant from LH332. It is contemplated that such a hybrid genetic complement can be prepared using *in vitro* regeneration of a tissue culture of a hybrid plant of this invention.

A hybrid genetic complement contained in the seed of a hybrid derived from LH332 is a further aspect of this invention. The genetic complements of a hybrid may be assessed by any of the many well known techniques as described above.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of the foregoing illustrative embodiments, it will be apparent to those of skill in the art that variations, changes, modifications, and alterations may be applied to the composition, methods, and

in the steps or in the sequence of steps of the methods described herein, without departing from the true concept, spirit, and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

REFERENCES

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